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LETTER TO EDITOR

Cross-validation of Liquid Chromatography-Tandem Mass Spectrometry Method for Quantification of Levofloxacin in Saliva

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Levofloxacin belongs to the Group A drug for treating multi-drug resistant tuberculosis (MDR-TB) but exhibits considerable pharmacokinetic variability. For a 750-1000 mg once daily dosing, the desired levofloxacin plasma/serum concentration range is 8-12 mg/L and the area under the concentration time curve from 0 to 24h is 75 if MIC is 0.5 mg/L and 150 if MIC is 1 mg/L. Saliva too could be a potential patient friendly alternative sampling matrix for levofloxacin quantification [1,2]. However, levofloxacin quantification in saliva using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method developed for plasma or serum requires cross validation. Moreover, the handling of infectious saliva samples from TB patients puts health care workers at risk of contagion. Membrane filtration was found to be suitable for sterilization of saliva samples [3]. The aims of this study were: a) to assess if drug concentrations in human saliva could be reliably determined with calibration samples prepared in human serum; and b) to perform a recovery test for levofloxacin concentrations in saliva after using sorbent material such as cotton rolls and/or filtering through a membrane filter.

A slight modification was done to our previously published LC-MS/MS method for levofloxacin quantification in human serum/plasma [4]. The assay was adjusted to simultaneously detect ciprofloxacin, moxifloxacin and levofloxacin in plasma/serum. First, for cross validation, levofloxacin stock solution of 2.5 mg/mL was prepared in dimethyl sulfoxide (Merck, NJ, USA). Nine different concentrations of the calibration samples in blank human serum were made: 0.20, 0.50, 1, 2, 5, 10, 20, 40, and 50 mg/L. In addition, four different concentrations of quality control samples (QC) in saliva, with a lower limit of quantification at 0.2 mg/L, low QC at 1 mg/L, medium at 20 mg/L, and high at 40 mg/L were prepared. The internal standard solution was prepared from a 1 mg/ml stock solution of [$^2H_4$]-levofloxacin in DMSO by diluting 50 µl to 250 ml with methanol (0.2 mg/L). For cross validation, all samples were analyzed in quintuplicate. The analysis was performed on a triple quadrupole LC-MS/MS (Thermo Scientific TSQ Quantiva, San Jose, CA, USA). A Thermo Accucore C18 analytical column of particle size 2.6 µm, 50 mm length, and internal diameter of

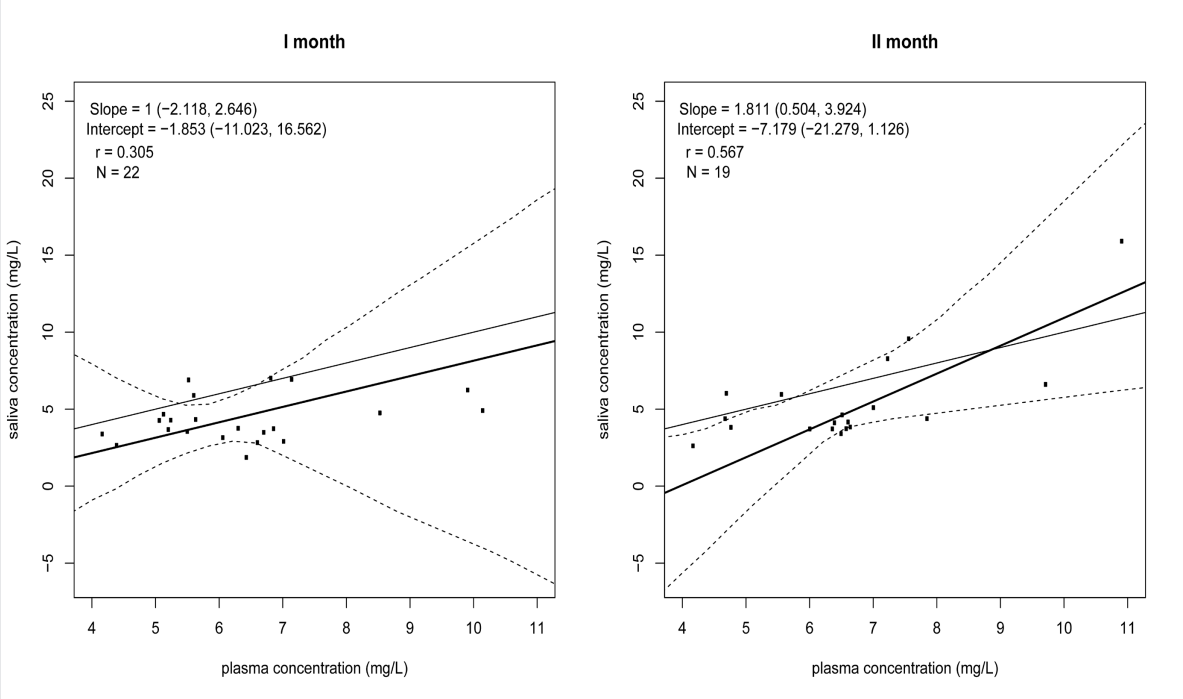

Figure 1. Passing-Bablok regression analysis of mean Lfx concentrations (at 0, 1, 2, 4, and 8 h) in plasma and saliva for 2 months. The bold solid line represents the Passing-Bablok fitted line, whereas the solid lighter line is the line of identity. The dashed lines indicate the 95% CI, r is the Spearman's rank correlation, and N is the number of paired mean plasma and saliva concentrations. Reprinted with permission from [5]. ©American Society for Microbiology (2019).

2.1 mm was used. The column temperature during analysis was 60°C. The linearity of the calibration curve was 0.20-50 mg/L for levofloxacin in both serum and saliva. QC samples in saliva at four concentration levels (0.20, 1, 20, 40 mg/L) were quantified using a calibration curve in serum. All QC samples were prepared and measured in 5-fold during a single day. The LC-MS/MS method had a run time of 2 min and levofloxacin eluted at a retention time of 0.7 min. Accepted bias and coefficient of variation (CV) were ≤15% for QC samples at low (at -0.9% and 1.0%), medium (at -0.3% and 0.9%), and high (at 2.0% and 1.3%) concentrations and ≤ 20% for LLOQ (at -1.0% and 2.3%) in saliva. This method was clinically applied for the analysis of levofloxacin concentrations in saliva samples at the laboratory of the department of Clinical Pharmacy and Pharmacology in the University Medical Center Groningen for a clinical trial (identifier number NCT 03000517) on the pharmacokinetics of levofloxacin in saliva of 23 MDR-TB patients. The median observed AUC_{0-24} and C_{max} in saliva were 67.09 mg*h/L and 7.03 mg/L [5]. Levofloxacin concentrations in plasma and saliva of 23 MDR-TB patients is shown (**Figure 1**).

Second, the recovery of levofloxacin in saliva was evaluated using four different solutions. The first group (blank syringe), was blank saliva which was absorbed by the cotton roll and afterwards compressed in a syringe. The effluent was then spiked with levofloxacin at 1 and 5 mg/L. In the second group (test solution syringe), levofloxacin spiked saliva at concentrations of 1 mg/L and 5 mg/L were applied to the cotton rolls. The volume required to saturate the cotton rolls was determined beforehand. Thereafter, cotton rolls with absorbed spiked saliva were compressed in a syringe by pushing the plunger of the syringe and collecting the effluent. The recovery was evaluated in the effluent using the

blank syringe solution as a reference. The third group (blank syringe filter) was similar to the first group, except the blank saliva was pushed through the syringe equipped with a 0.22 µm polyvinylidene fluoride membrane filter, and later spiked with levofloxacin at the above-mentioned concentrations. In the fourth group (test solution syringe filter), recovery yield was determined after compressing fully saturated cotton rolls with levofloxacin spiked saliva at (1 mg/L and 5 mg/L) in a syringe equipped with a 0.22 µm membrane filter. The blank syringe filter solution was used as a reference to determine the recovery. Our study has shown that the plain cotton rolls achieved a recovery of around 70% at 1 mg/L with a CV% of 9.5%; whereas at 5 mg/L the mean recovery was more variable between the groups (63-80%) with a CV of 6.0%. This will have an impact on the variability of analytical results with a spread of 17% and bias of approximately 30%, if cotton rolls are used as a sampling device. This is likely due to sorption of levofloxacin to the cotton roll. Therefore, saliva samples could be useful only in screening and semi-quantitative prediction of plasma levels of anti-TB drugs [5]. In addition, our experiments have shown that filtration through a 0.22 µm polyvinylidene fluoride membrane filter does not result in a further loss of levofloxacin.

In conclusion, results of cross-validation study were within the acceptance criteria for bias and precision according to formal regulations. The cotton rolls used for saliva sample collection achieved a levofloxacin recovery of around 70%.

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